CYTOKINESIS OF MULTI-SPINDLE CELLS

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Although in cell division it is usually apparent that the position of the constriction furrow is related to that of the spindle, the relationship of the two is not clear. Harvey (1935), working with centrifuged sea urchin eggs, came to the conclusion (p. 296) that, "Since a cleavage plane can come in without relationship to the mitotic figure, it would seem that the mitotic figure itself is an expression of more fundamental physical and chemical changes taking place in the cytoplasm and surface of the egg, and not a cause in itself of the cleavage process." Waddington (1952), working with frog eggs, stated (p. 488) that, "The only evidence, in the present experiment, suggesting an influence by the spindle on the cleavage furrow is that of the few eggs described in which an attempt to move the spindle was followed by an alteration in the course of the furrow. General considerations derived from a study of the cleavage of eggs of many groups certainly suggest that the location of the spindle ultimately determines the position in which the furrow appears." Carlson (1952), on the basis of microdissection studies on grasshopper neuroblasts, held (p. 217) that, "Formation of the cleavage furrow is independent of the spindle and chromosomes, but its position depends to a limited extent on their position at late anaphase." The writer (Roberts, 1955), working with the same material, has presented evidence suggesting the spindle poles, through their relationship to expansions of the polar cell membranes, play an important part in determining the site of the constriction furrow. Cornman and Cornman (1951) have suggested that, upon the dissolution of the nuclear membrane, nuclear material gathers around the centrosomes, is transported by the asters to the cell surface, and is carried by currents to the equator, where it induces the establishment of the furrow. Marsland (1939) and others have described the existence of a "plasma-gel girdle" around the equator of the sea urchin egg prior to cytokinesis without relating it in any way to the spindle.

Cells containing several spindles, usually two to four, are regularly found in preparations of isolated living testicular cells of *Popilius disjunctus*. As is usual in this material, the spindles are invested by long filamentous mitochondria from pole to pole, making the spindles strikingly visible, especially with phase microscopy. Such a multi-spindle condition has been observed in spermatogonia and primary and secondary spermatocytes, which are considerably larger than their single spindle counterparts. Conditions in these cells differ from those seen in dispermic and parthenogenetic marine eggs in that they arise from regularly occurring multinucleate cells, and, ordinarily, each spindle has its own centrosomes and chromosome complement. During cytokinesis each cell divides into two or more cells, de-

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pending on the number of spindles and the orientation of the spindles with respect to each other and to the cell membrane. The patterns of cytokinesis which are observed with varying spindle orientations provide a means for studying the role of the spindle poles in the establishment of constriction furrows.

Materials and Methods

Beetles were collected from rotting hardwood logs, brought into the laboratory, and maintained by supplying moist pieces of wood from the logs where they were collected. During the colder months best results were obtained from beetles which had been kept at room temperature, 22° C., for at least one week. Prolonged exposure to temperatures above 30° C. resulted in an absence of dividing cells in the testes. With proper temperature and humidity conditions, dividing cells may be obtained during the entire year.

Several media have been used for the culture of isolated beetle cells. Whole hemolymph is unsatisfactory because it rapidly darkens and becomes cytolytic upon exposure to air. The addition of purified phenyl thiourea prevents blackening and reduces the cytolytic effect, but cell division is adversely affected. A better medium is obtained by flash heating hemolymph, which inactivates those factors responsible for blackening and cytolysis, but also coagulates and precipitates proteins. Cells suspended in such deproteinated hemolymph divide fairly well, but the preparations do not last as long as is desirable. The modified insect Tyrode solution used by Gaulden and Carlson (1951) is satisfactory for preparations which are to be studied for short periods of time, 4 to 6 hours. Best results have been obtained by using a mixture of the insect Tyrode solution with deproteinated hemolymph. The proportions are not critical; usually 50% to 75% deproteinated hemolymph has been used. Preparations made with this medium usually last 18 to 24 hours, and permit observation of two complete cycles of division.

The preparation of this medium presents several difficulties and therefore will be described in some detail. A sterile one-ml. tuberculin syringe equipped with a 1½ inch 26-gauge needle and containing 0.05 ml. of insect Tyrode solution is held in readiness. A beetle, preferably male, is selected and the anterior portion of the head, at the level of the dorsal horn, cut off by one swift cut with heavy scissors. Hemolymph from the severed dorsal vessel accumulates in large clear drops. If the body of the beetle is not squeezed, there is usually no contamination of the hemolymph by material from the digestive tract. If contamination does occur, it can be detected by a brown color in the otherwise colorless fluid, in which case it must be discarded. Occasionally, compression of tissue blocks the dorsal vessel, but this can usually be overcome by slight probing with the syringe needle. The drops of hemolymph are drawn into the syringe and mixed with the Tyrode solution. Ordinarily, enough hemolymph can be obtained to make the total fluid volume in the syringe up to 0.15 ml. It is essential to work rapidly, since the blackening occurs within a few minutes and renders the fluid useless. The fluid in the syringe is discharged into a thin-walled glass bubble for flash heating. The bubbles used are blown from Pyrex tubing and are about 15 mm. in diameter with a capillary neck about 20 mm. in length. The diameter of the neck is just sufficient to admit the 26-gauge needle. After introduction of the fluid, the tip of the neck is sealed

PLATE I

FIGURES 1–20.

in a flame and the bubble is plunged into hot water, just below the boiling point, for 45 seconds. It is convenient to have a beaker of boiling water available and to remove it from the heat while the bubble is being sealed. The fluid within the bubble must be agitated as it enters the hot water and during the entire heating process, otherwise, it will gel and no free fluid can be obtained. In successful preparations the precipitated proteins adhere to the wall of the bubble. After cooling, the bubble should be rotated so that any droplets of distillate are incorporated into the fluid.

Two pairs of testes are present in the adult male beetle. Ordinarily, one pair will supply sufficient material for the amount of medium available. The testes are dissected out and placed in a small dish of Tyrode solution and adherent tissue removed. It is important that the seminal vesicle be removed, otherwise the preparation will be filled with spermatozoa. Nematodes, which are abundant during the summer months, should be removed. The Tyrode solution also has the important function of washing the testes free of unaltered hemolymph which, if carried into the final preparation, has a deleterious effect. The testes are transferred from the Tyrode solution to the center of a sterile microscope slide, carrying over as little fluid as possible. They are then covered with about half the prepared medium, which has been withdrawn from the bubble with a sterile one-ml. tuberculin syringe and a 26-gauge needle. The testes are squashed against the slide, using flat-tip cover-slip forceps. Thorough squashing is necessary to rupture the cysts of the testis, but a scouring motion, which destroys many cells by its shearing action, should be avoided. The remainder of the medium is discharged over the squashed testes and the slide tilted so that the fluid and cells drain away from the squashed tissue. This cell suspension is then drawn into the same syringe, leaving the fibrous debris on the slide.

One-centimeter squares are outlined on coverslips with vaseline extruded from a 26-gauge needle. Each square is flooded with the prepared cellular suspension

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FIGURES 1–4. Division of a secondary spermatocyte containing two parallel spindles. A normal spermatid containing a single nebenkern (nb) is shown at the lower left of Figure 2. The two large spermatids resulting from the division are shown in Figure 4.

FIGURES 5–7. Division of a secondary spermatocyte containing two divergent spindles. A well developed achromosomal spindle (ach) connected the divergent poles. A second furrow formed between the divergent poles but soon regressed. Two large spermatids resulting from the division are shown in Figure 7.

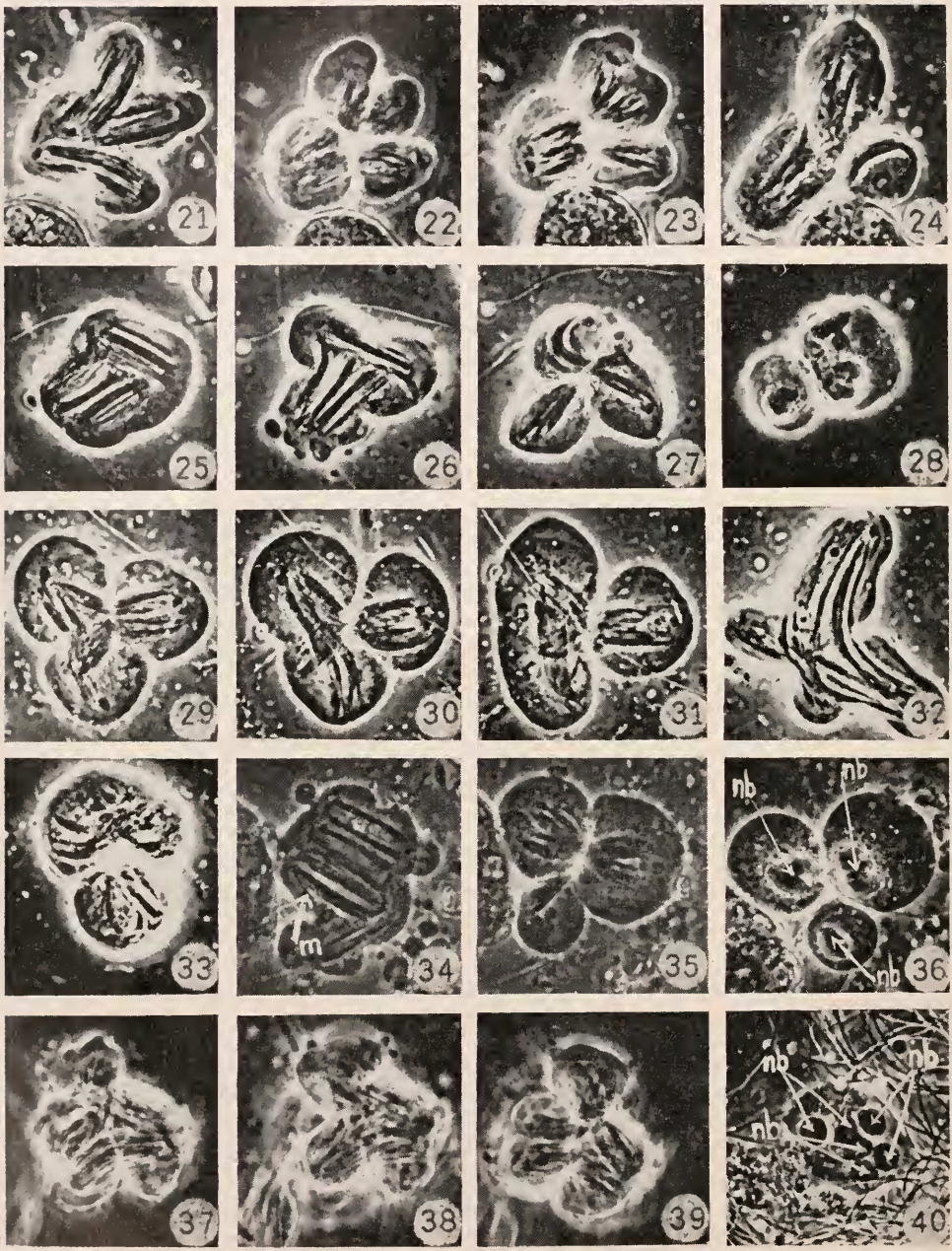
FIGURE 8. Three daughter cells resulting from division of a primary spermatocyte containing two divergent spindles.

FIGURES 9–11. Division of a primary spermatocyte containing two divergent spindles. There was no evidence of cross-connections between the divergent poles. A furrow developed between the divergent poles and advanced halfway before regressing.

FIGURES 12–16. Division of a primary spermatocyte containing two spindles at right angles to each other, in different planes and not intersecting. There was no evidence of cross-connections between the poles. Furrows developed between the poles and the cell appeared to be dividing into four. As the furrows impinged on the spindles their orientation changed so that they came to be approximately parallel and the cell divided into two.

FIGURES 17–20. Cytokinesis of a primary spermatocyte (?) containing three spindles. Furrows developed between the poles, in the equatorial region. In late cytokinesis one furrow regressed, and two of the daughter cells fused. In Figure 20 it can be seen that in one case the two chromosome groups fused, forming a large nucleus (N) and in another the groups remained separate, forming two small nuclei (n).

PLATE II



FIGURES 21–40.

and the excess fluid withdrawn into the syringe. This gives a relatively flat film whose thickness can be adjusted as required. The coverslips are inverted over depression slides and sealed with vaseline or heavy mineral oil. Two types of depression slides have proved especially useful. One (Fisher-Littman) is of the same thickness as a standard microscope slide and has a shallow circular depression with a flat polished bottom. This slide permits the use of oil immersion phase objectives. A specially made plastic well slide has been most commonly used. This slide is too thick to permit the use of oil immersion objectives, but has the advantage that it takes 22 × 50 mm. cover-slips on which three vaseline squares can be outlined. The relatively large air space is compensated for by recesses on all four sides into which cotton, wet with Tyrode solution, can be packed.

Divisions were studied with dark contrast phase optics (Spencer) using long focal length phase plates, to permit study of thick preparations. In the writer's experience with living cells, the long focal length phase plates have proved to be clearly superior, regardless of the thickness of the preparation. Light intensity was controlled by a variable transformer and overheating of the slides was avoided by using a copper sulfate water cell and three heat-absorbing filters (Corning). Observations and records were made at room temperature, about 22° C.

Observations

The observations reported are based on spindle orientation in living cells as revealed by oriented filamentous mitochondria. The spindle fibers are not visible. A comparison with stained sections indicates that the structure observed in living cells is a fairly accurate representation of the actual distribution of fibers. The chromosome-bearing spindles are faithfully visualized, but cross-connections (achromosomal spindles) between the poles of adjacent chromosome-bearing spindles are less reliably indicated. The filamentous mitochondria, which are preserved and stained by those methods used to demonstrate fibers, tend to obscure the fibers along which they are oriented. Wherever oriented mitochondria indicate the existence of such cross-connections, fiber connections actually exist, and the relative abundance of fibers is correlated with the abundance of oriented mitochondria. In the absence of oriented mitochondria, a few weak fibers may be present or there may be none.

Figures 21–24. Cytokinesis of primary spermatocyte containing three divergent spindles. There was no evidence of cross-connections between the divergent poles. Furrows developed between the divergent poles, and the cell appeared to be dividing into four. Strong blebbing, Figures 22, 23, was followed by regression of one furrow, and the cell eventually divided into three.

Figures 25–28. Cytokinesis of a secondary spermatocyte containing three spindles. After cytokinesis was virtually complete, Figure 27, two daughter cells fused, giving rise to one large and one small spermatid.

Figures 29–33. Cytokinesis of primary spermatocytes(?) with three spindles.

Figures 34–36. Cytokinesis of a secondary spermatocyte containing four spindles, three parallel and the fourth divergent. Although a mitochondrion (m) was present in the area between the divergent poles, no achromosomal spindle was visible. Two large and one small spermatid resulted. Note the relative size of the nebenkerne (nb).

Figures 37–39. Cytokinesis of a cell containing six spindles. Three daughter cells resulted.

Figure 40. Spermatid containing six nebenkerne (nb). This cell was observed immediately after the preparation was made, far too short a period for this to be the result of the procedure used.

Figures 1–16 illustrate cytokinesis of cells containing two chromosome-bearing spindles. In the simplest situation the two spindles, each with its own chromosome complement, were parallel and close together. The furrow formed in the equatorial position and the cell constricted into two daughter cells as though only one spindle were present (Figs. 1–4). In some cases the two chromosome groups in each polar region fused to form one nucleus. In others, two nuclei were formed. In many double spindle cells the spindles were in the same plane but lay at an angle to each other. Under these conditions a furrow developed in the equatorial region, and a second furrow invariably developed between the divergent poles. The formation of the second furrow did not depend upon the presence of cross-connections between the divergent poles, as far as could be judged from the presence of oriented mitochondria. In some cases a well developed achromosomal spindle was present (Figs. 5–6), and in others (Figs. 9–10) there was little or no evidence of cross-connections. The fate of the second furrow depended primarily upon the distance between the divergent poles and not on the presence of fiber connections between these poles. Where the poles were only slightly separated, the second furrow soon regressed and the cell constricted into two daughter cells (Figs. 4, 7). Regression often occurred after cytokinesis was virtually complete, resulting in the fusion of two or even all three of the daughter cells. A few double-spindle cells have been observed in which the two spindles lay at right angles to each other, in different planes, and not intersecting (Figs. 12–16). During cytokinesis furrows developed between each of the four poles so that the cell appeared to be dividing into four daughter cells. In the cell illustrated there was no evidence of cross-connections between the poles. In cells of this type, as the furrows impinged upon the spindles their orientation changed so that they came to be approximately parallel, and the cell eventually divided into two.

Figures 17–33 illustrate cytokinesis of cells containing three chromosome-bearing spindles. Various spindle orientations occurred. In some, the three spindles lay in the same plane and formed the sides of an equilateral triangle (Fig. 17). Furrows developed in the equatorial region of each spindle and developed so that division into three daughter cells was virtually completed (Fig. 19). In late telophase, after re-formation of interphase nuclei, two of the daughter cells fused into one large cell (Fig. 20). In another case, first observed in early cytokinesis, the three spindles were arranged as shown in Figure 21. There was no evidence of cross-connections between adjacent poles. Furrows developed between each of the diverging poles and across the equatorial region of all three spindles. The cell appeared to be dividing into one large and three smaller daughter cells. Later, strong blebs developed on two adjacent incipient daughter cells (Figs. 22, 23), the furrow between them receded, and the cell finally divided into two large and one small daughter cell (Fig. 24). It is noteworthy that the appearance of strong blebs adjacent to a furrow always resulted in the regression of that furrow. In another case two of the three spindles were parallel and the third at approximately right angles to the other two (Fig. 25). Furrows formed in the equatorial regions and advanced until, in late cytokinesis, two incipient daughter cells fused so that one large and one small daughter cell were produced (Fig. 28). Other spindle orientations in which the spindles were, in part, in different planes are illustrated in Figures 29–31 and 32–33. As in the other cases described, furrows developed between

the divergent poles without regard to the presence or absence of cross-connecting fibers.

Cells containing from four to six chromosome-bearing spindles have been observed. Cytokinesis in such cells is complex and difficult to follow (Figs. 37–39). A simpler case of a cell with four chromosome-bearing spindles is illustrated in Figures 34–36. It is apparent here also that furrows develop between spindle poles and not necessarily in relationship to the equatorial region of the cell or spindle.

Discussion

It is evident in this material that the site of furrow formation is related to, and apparently dependent upon, the position of the spindles. It has been repeatedly observed that furrows form approximately halfway between spindle poles and not necessarily in relation to the equators of the spindles. The presence of spindle fiber tracts (chromosomal or achromosomal spindles) between the poles does not appear to be essential for the formation and further development of a furrow. Since in the usual situation the equator of a spindle does lie halfway between spindle poles, the commonly observed relationship between furrow and spindle equator can be explained on this basis.

Bajer (1954) has described a quite similar situation in primary spermatocytes of the snail *Cepea hortensis*. Although his figures of trinuclear and tetranuclear cells show only the chromosome groups and not the spindles, it is clear here also that furrows form between spindle poles and not in relationship to the equators. His description of cytokinesis of binuclear cells without division of the nucleus is especially striking. In these cells each nucleus reached metaphase, with the two spindles parallel and widely separated. After a "long" metaphase with "quiverings" of the plate, the furrow formed and advanced between the two spindles without cutting through either. The cell divided into two and interphase nuclei were reconstituted from each metaphase configuration.

These observations support the idea that the spindle poles initiate furrow formation, possibly through a "furrow determiner" as postulated by Cornman and Cornman (1951), or a "structural agent" as proposed by Swann (1952) and Mitchison (1952) and modified by Roberts (1955). They do not appear to provide an adequate basis for deciding between these alternatives. Bajer's observations on cytokinesis of binuclear cells without nuclear division would appear to support the view of the Cornmans that nuclear material released upon the dissolution of the nuclear membrane may act as a furrow determiner. It has been noted that in multi-spindle cells the distance apart of divergent poles influences the furrow which develops between them. When the distance is sufficiently great, the furrow advances and may succeed in dividing the cell. This does not appear to support the view of the Cornmans, since it would seem that the accumulation of large quantities of "furrow determiner" in the small area between the poles should result in the development of a strong furrow. Swann and Mitchison postulate that the furrow is established by an initial contraction of the cell membrane between the "structural agent" induced polar membrane expansions, and that spread of the expansions into the furrow actively advances it. In this material it has been observed that when large blebs, which are membrane expansions of a sort, occur in the furrow area,

the furrows usually regress. Further, it would seem that proximity of a developing furrow to the point of release of the expansion inducing substance should facilitate the further growth of the furrow. There is no difficulty if the furrow, after a critical stage in its establishment, is autonomous and continues to develop by contraction or autocatalytic growth as suggested by the Cornmans. This is supported by the work of Waddington (1952), Harvey (1935), Carlson (1952) and others. The observation that the furrow developing between spindle poles separated by only a short distance soon regresses could be explained as the result of the initial contraction being obliterated by the approaching waves of expansion before the critical stage is reached.

In the present work, as in that of the Cornmans, there is evidence of a critical stage just at the completion of cytokinesis. Where multiple furrows are present, it is usual that one or more furrows regress, and it is rarely that these cells succeed in dividing into more than two daughter cells. This was also true of the multinuclear cells described by Bajer. Certainly adequate spindle material is available, and it is not likely that this is a factor as suggested by the Cornmans. Bajer's observations indicate that spindle material is not required for the completion of cytokinesis, since constriction was completed without the furrow cutting through a spindle.

It is evident that some unusual chromosomal relationships must result from divisions such as those described. These have not thus far been analyzed. Preliminary attempts to do chromosome counts by the squash method have not been satisfactory. The chromosomes are so small that the pressure required to spread them sufficiently for counting made cell boundaries unrecognizable, and it was not possible to determine whether adjacent chromosome sets came from the same or different cells.

The multi-spindle cells studied do not result from the procedures used. Prometaphase lasts approximately three hours, and anaphases and telophases have been observed within a few minutes after making a preparation. Living spermatids have frequently been observed to receive as many as two sets of chromosomes (Figs. 4, 7, 28) and occasionally as many as three (Fig. 36). In the normal spermatid the single nebenkern divides into two before the mulberry stage is reached, so that not more than two nebenkerne are normally seen. Spermatids containing at least six nebenkerne (Fig. 40) have been observed in freshly made preparations.

Summary

1. Patterns of cytokinesis have been studied in living multi-spindle cells obtained from beetle testes.

2. Furrows develop between spindle poles, whether or not connected by spindle fibers, and not necessarily in relationship to the equator of a spindle.

3. The further development of furrows is related to the distance apart of spindle poles. If the spindle poles are close together, the furrow soon regresses; if the poles are sufficiently far apart, the furrow advances and may succeed in dividing the cell.

4. When large blebs develop in the furrow area, the furrow regresses.

5. The observations reported are discussed in the light of current theories of cytokinesis.

6. Details of the unusual chromosome distributions which result have not been analyzed in detail.

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